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Histone Deacetylase Inhibitor Stimulate *CYP3A4* Proximal Promoter Activity in HepG2 Cells

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The expression of *CYP3A4* gene is induced by a variety of structurally unrelated xenobiotics including the antibiotic rifampicin, pregnenolone 16-carbonitrile (PCN), and endogenous hormones, that might mediate through steroid and xenobiotic receptor (SXR) system. The molecular mechanisms underlying regulation of *CYP3A4* gene expression have not been understood. In order to gain the insight of the molecular mechanism of *CYP3A4* gene expression, study has been undertaken to investigate if the histone deacetylation is involved in the regulation of *CYP3A4* gene expression by proximal promoter in human hepatoma HepG2 cells. Also we have investigated to see if SXR is involved in the regulation of CYP3A4 proximal promoter activity in human hepatoma HepG2 cells. HepG2 cells were transfected with a plasmid *pCYP3A4-Luc* containing~1 kb of the *CYP3A4* proximal promoter region (-863 to +64 bp) in front of a reporter gene, luciferase, in the presence or absence of pSAP-SXR. In HepG2 cells, CYP3A4 inducers, such as rifampicin, PCN and RU486 showed minimal stimulation of *CYP3A4* proximal promoter activity in the absence of SXR and histone deacetylase (HDAC) inhibitors. 4-Dimethylamino-*N*-[4-(2-hydroxycarbamoylvinyl)benzyl]benzamide (IN2001), a new class HDAC inhibitor significantly increased *CYP3A4* proximal promoter activity over untreated control cells and rifampicin concomitant treatment with IN2001 increased further *CYP3A4* proximal promoter activity that was stimulated by IN2001. The results of this study demonstrated that both HDAC inhibitors and SXR are essential to increase of *CYP3A4* proximal promoter activity by CYP3A4 inducers such as PCN, rifampicin, and RU486. Especially SXR seems to be important for the dose dependent response of CYP3A4 inducing chemicals to stimulate *CYP3A4* proximal promoter activity. Also this data suggested that HDAC inhibitors seemed to facilitate the *CYP3A4* proximal promoter to be activated by chemicals.

Key words: *CYP3A4,* PCN, Rifampicin, RU486, SXR, HDAC, IN2001, HepG2

INTRODUCTION

CYP3A4 is involved in the metabolism of an extensive range of endogenous steroids and xenobiotics, making a significant contribution to the termination of the action of steroid hormones, elimination of foreign chemicals and activation of several potent carcinogens. It has been estimated that about 60% of currently marketed drugs are metabolized by CYP3A4 (Michalets *et al.*, 1998; Guengerich *et al.*, 1999; Li *et al.*, 1995). The substrates for this enzyme include drugs such as quinidine, nifedipine, diltiazem, lidocaine, lovastatin, erythromycin, cyclosporin, triazolam, midazolam and endogenous substances, including testosterone, progesterone, androstenediol and bile acids (Guengerich *et al.,* 1999). CYP3A4 also activate procarcinogens, including aflatoxin B1, polycyclic aromatic hydrocarbons (PAHs), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 6-aminochrysene (Guengerich *et al.*, 1999; Pascussi *et al.*, 2003). *CYP3A4* is transcriptionally regulated by a variety of hormones, including glucocorticoids, growth hormone, and triiodothyronine, and xenobiotics such as phenobarbital, clotrimazole, mifepristone (RU-486), phenytoin, phenylbutazone, omeprazole, paclitaxel, spironolactone, cyproterone acetate and rifampicin (Schuetz *et al.*, 1993; Liddle *et al.*, 1998; Kocarek *et al.*, 1995). Rifampicin, a macrocyclic antibiotic, is known to be one of the most potent inducers of *CYP3A4* expression both *in vivo* and *in vitro* (Schuetz *et al.,* 1993; Kocarek *et al.*, 1995; Michalets *et al.*, 1998). In addition, CYP3A4 activity is inhibited by

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several clinically used drugs such as erythromycin, gestodene and ketoconazole (Ketter *et al.*, 1995). This diverse range of substrate, inducers and inhibitors of CYP3A4 activity creates the potential for clinically significant drug interactions. It is, therefore, evident that a basic understanding of how the *CYP3A4* gene is regulated is of considerable relevance to drug development and homeostasis as well as clinical medicine (Ogg *et al.*, 1999; Ketter *et al.*, 1995).

The *CYP3A4* gene is 27 kb long with 13 exons (Guengerich *et al.*, 1999). The molecular mechanism which is based on the regulation of *CYP3A4* gene expression has been studied by several groups. Initially, 1105 bp of proximal promoter was isolated and computer analysis showed the presence of several putative transcription factor binding sites including the glucocorticoid response element (GRE), estrogen responsive element (ERE), chicken ovalbumin upstream promoter-transcription factor (COUP-TF), HNF-4, HNF-5, p53, octamer transcription factor-1 (Oct-1) and a basal transcription element (-35 to -50 bp) (Hashimoto *et al.*, 1993; Itoh *et al.*, 1992). Although the nucleotide sequence of the proximal 5'-flanking region of *CYP3A4* has been reported, the molecular mechanism underlying the transcriptional regulation of this gene has yet to be elucidated. The proximal promoter of the *CYP3A4* gene (172 to 149 bp) contains two copies of an AG(G/T)TCA hexamer, the recognition sequence for the nuclear receptor family of transcription factors (Mangelsdorf *et al.*, 1995). Barwick *et al.* demonstrated that these half-sites in proximal promoter, organized as ER-6 (everted repeat separated by six nucleotides, 5'-TGAACTcaaagg AGGTCA-3'), conferred rifampicin responsiveness on heterologous reporter gene constructs when transfected into rabbit hepatocytes (Barwick *et al.*, 1996). Goodwin *et al.* reported that distal enhancer module (*XREM,* xenobiotic-responsive enhancer module) is located -7800 bp upstream of the *CYP3A4* transcription initiation site and enhances the nuclear receptor mediated-transactivation of *CYP3A4* (Goodwin *et al.*, 1999). This *XREM* region also includes at least two elements that are capable of binding nuclear receptors, which are DR-3 (directed repeat separated by three nucleotides, 5'-TGAACTtgcTGACCC-3') and ER-6 (5'-TGAAATcatgtcGGTTCA-3'). DR-3, DR-4, ER-6 and ER-8 are recognized by SXR, CAR and the vitamine D receptor (VDR) after heterodimerization with RXR (Sueyoshi *et al.*, 1999; Drocourt *et al.*, 2002; Thummel *et al.*, 2001). These observations suggest that these receptors are capable of regulating same series of genes through the same *cis*-acting elements, and that cross-talk between these signalling pathways is an important factor in mounting an appropriate response to a xenobiotic challenge. The regulation of native *CYP3A4* proximal promoter activity was not understood. Recently it has been reported that histone deacetylation is related with the expression of *CYP1A* and *CYP1B* (Nakajima *et al.*, 2003).

Therefore the present study was aimed at investigating the role of the histone deacetylation on the regulation of *CYP3A4* gene expression by proximal promoter in the presence or absence of SXR. HepG2 cells were transfected with a plasmid containing ~1 kb of the *CYP3A4* proximal promoter region (-863 to +64bp) which was cloned in front of luciferase gene as a reporter, in the presence or absence of SXR. Transfected cells were treated with various chemicals such as rifampicin, PCN and RU-486, in the presence or absence of TSA and then cells were lysed and assayed for the luciferase activity using luciferin.

MATERIALS AND METHODS

Materials

Rifampicin (Rif), RU-486 (RU), pregnenolone 16α-carbonitrile (PCN), HC-toxin was purchased from Sigma-Aldrich (St. Louis, MO). Agarose was purchased from FMC and LipofectAMINE from Gibco-BRL and *Hind III*, *Sma I* from BMS, while pGL3 basic vector and luciferase assay system were ordered from Promega. Trichostatin A, 4-dimethylamino-*N*-[4-(2-hydroxycarbamoylvinyl) benzyl] benzamide (IN2001) and IN2002 were synthesized.

Plasmids

phCYP3A4-Luc

The chimeric *CYP3A4* luciferase reporter plasmid, *phCYP3A4-Luc*, was constructed in our laboratory from *phCYP3A4-lacZ*. The *hCYP3A4* promoter, from base pairs -863 to +64, was generated by polymerase chain reaction (PCR) using the DNA template isolated from the human lymphocyte. The PCR sense and antisense primers used were 5'-CATGCCCTGTCTCTCCTTTAG-3' (corresponding to nucleotides-863 to -843) and 5'-CCTTTCAGCTCTGTG TTGCTC-3' (corresponding to nucleotides +44 to +64), respectively. The amplification cycle profile included denaturation at 93°C for 1 minute, annealing at 54°C for 2 minutes, and extension at 72°C for 1 minute. The product (928-bp) was then subcloned into the *pGEM-T* vector, and the resulting construct digested with *EcoR I* to obtain a 943-bp, with additional 13-bp, cloning restriction eyzyme site sequence. The 943-bp fragment was then cloned into the *EcoR I Sal I* site in the 5' to 3' orientation with respect to the *lacZ* transcription unit in the *pCMV* plasmid (*phCYP3A4-lacZ*). For *phCYP3A4-Luc*, *phCYP3A4-lacZ* was digested with *EcoR II* and was prepared *CYP3A4* insert DNA containing ~1 kb of *CYP3A4* proximal promoter. *pGL3-basic* vector, a promoter-less luciferase reporter vector, was linearized with *Sma I*. *CYP3A4* insert DNA was cloned into a linearized *pGL3-basic* vector at *Sma I* site. Finally, *phCYP3A4-Luc*, *CYP3A4* luciferase reporter

plasmid, containing ~1 kb (863 to +64 bp) of the *CYP3A4* proximal promoter was prepared.

pSAP-SXR

The pSAP-SXR contained SXR cDNA, under the control of the liver-specific human SAP promoter. The SXR gene sequence was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) with a full-length of mRNA isolated form HepG2 cells. The primers used for the amplification were the SXR sense and antisense primers, 5'-CAAGGACAGCAGCATGACAGTC-3' (corresponding to nucleotides +36 to +57 bp) and 5'-GCTCATCTACCTGT GATGCCGAA-3' (corresponding to nucleotides +1472 to +1450 bp), respec-tively. The amplified SXR was 1437 bp in length, which was then cloned into the *pGEM-T* vector. The pGEM/SXR construct was digested with *EcoR I* to clone it into the *EcoR I* site of *pBluscript SK*+ (pBS-SXR). Separately, the liver-specific human serum amyloid P component (SAP) gene promoter was purified from pLG I-SAP by digestion with *Hind III/BamH I*, and the SXR fragment then inserted into the site *Hind III/BamH I* of pBluscript SK+ (pBR-SAP). The pLG I-SAP plasmid was a gift Dr. Kenichi Yamamura of the Kumamoto University, Japan. Following digestion of pLG I-SAP with *Xho I/Spe I*, this fragment was inserted into the *Xho I/Spe I* cut *pTet-Splice* that had the tetracycline operator sequence eliminated by digestion with *Xho I/Spe I* enzymes (pSAP-Splice). Finally, the SXR fragment isolated from pBS-SXR with *Spe*was cloned into the *Spe* I site of pSAP-Splice downstream of the *SAP* promoter. The SXR sequence was linked to the SV40 polyadenylation signal of *pTet-Splice*.

Cell culture and transfection

HepG2 human liver carcinoma cell lines were grown in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% (v/v) fetal bovine serum and penicillin-streptomycin (100 U/mL). For the transfection of *phCYP3A4-Luc*, HepG2 cells were seeded in multi-well plates. *phCYP3A4Luc* and/or pSAP-SXR and LipofecTAMINE (Invitrogen) were mixed in serum-free medium and incubated at room temperature for 45 min before the addition to each well. Cells were incubated for at least 5 h before adding normal DMEM containing 20% fetal bovine serum. The details were followed as supplier's manual.

Chemical treatment

HepG2 cells were rinsed with PBS three-times before the administration of various chemicals. Stock solutions of chemicals were made in dimethylsulfoxide (DMSO) as a vehicle and control cells were treated with 0.1% DMSO or chemicals for 24 h. Final concentration of DMSO did not exceed 0.2%.

Luciferase reporter assay

Luciferase assays were performed using the Luciferase Assay System (Promega). Briefly, the transfected cells were lysed with reporter lysis buffer. The lysates was incubated with luciferase substrates, luciferin and luciferase activity was determined by the luminometer. Protein assay of cell extracts was carried out using the Micro BCA protein assay reagent kit (Pierce) and an ELISA Reader (Bio-rad). Luciferase activity was normalized to protein content. The data are presented as the fold induction of control cells that were treated with 0.1% DMSO.

RESULTS

CYP3A4 proximal promoter response to known CYP3A4 inducing chemicals

To investigate whether *CYP3A4* proximal promoter was regulated by CYP3A4 inducers such as rifampicin, PCN and RU-486, transient transfection experiments were performed in HepG2 cells. Reporter gene, *phCYP3A4-Luc,* containing 928 bp of the *CYP3A4* proximal promoter (-863 to +64 bp) was transfected. Then CYP3A4 inducers (0.25 to 25 μM) were treated for 24 hr and the activity of luciferase was measured. When PCN was treated with 0.25, 2.5 and 25 μM, the activity appeared to be slightly increased to 1.2-, 1.2- and 1.3-fold respectively compared to control (Fig. 1). RU-486 0.25, 2.5 and 25 μM increased the promoter activity slightly to 1.1-, 1.3- and 1.5-fold over control, respectively. But both RU486 and PCN did not show statistically significant change. These results indicated that CYP3A4 inducers did not increase the activity of *CYP3A4* proximal promoter.

Influence of SXR on the *CYP3A4* proximal promoter activity in response to CYP3A4 inducing chemicals

CYP3A4 expression by proximal promoter was progressively increased, when the increasing amount of cDNA expression plasmid of SXR was cotransfected with *phCYP3A4-Luc* (data not shown). Therefore, in order to examine whether SXR enhances further the activity of *CYP3A4* proximal promoter that was stimulated by CYP3A4 inducers, *phCYP3A4-Luc* was cotransfected with pSAP-SXR and then these cotransfected cells were treated with CYP3A4 inducer, rifampicin (0.25 to 25 μM) for 24 h. The activity of luciferase from these cells showed no increase of the proximal promoter activity of *phCYP3A4-Luc* as increased the concentrations of rifampicin compared to control (Fig. 2). These results indicated that SXR was unable to enhance rifampicin action on *CYP3A4* proximal promoter in terms of stimulation of gene expression.

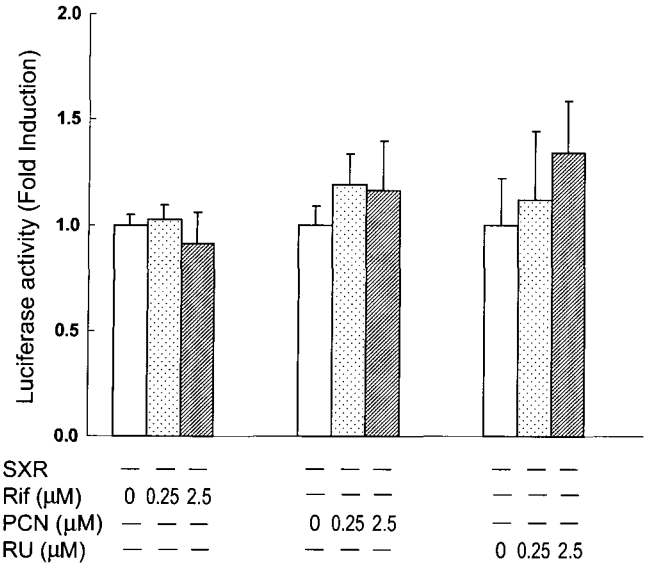

Fig. 1. Effects of CYP3A4 inducers on *CYP3A4* proximal promoter in *phCYP3A4-Luc* transfected HepG2 cells. Reporter genes containing 928 bp of the *CYP3A4* promoter (-863 to +64 bp) were constructed as described in *materials and methods.* HepG2 cells were transiently transfected with *phCYP3A4-Luc* and exposed to various concentrations of CYP3A4 inducers such as rifampicin (Rif), pregnenolone 16α-carbonitrile (PCN) and RU-486 (RU) for 24 h. Control cells were treated with 0.1% DMSO. The luciferase activity was measured using luciferin. Data present mean ± S.D. of triplicate transfections from a single representative experiment.
* : Significantly different from control at $p < 0.05$

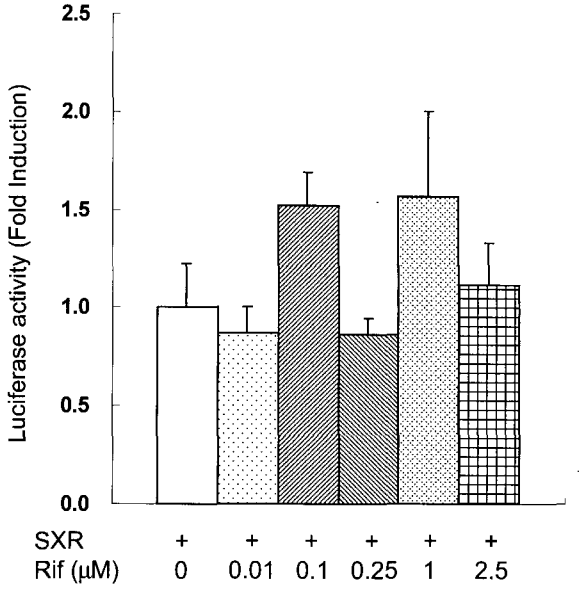

Fig. 2. Effects of CYP3A4 inducers on *CYP3A4* proximal promoter in *phCYP3A4-Luc*/SXR cotransfected HepG2 cells. HepG2 cells were transfected by *phCYP3A4-Luc* along with pSAP-SXR. Cells were exposed to various concentrations of rifampicin (Rif) for 24 h. Control cells were treated with 0.1% DMSO. The luciferase activity was measured using luciferin. Data present mean ± S.D. of triplicate transfections from a single representative experiment.

Effects of SXR, HDAC inhibitor and CYP3A4 inducer on *CYP3A4* proximal promoter in HepG2 cells

Effects of SXR and CYP3A4 inducer on *CYP3A4* proximal promoter in *phCYP3A4-Luc*/pSAP-SXR cotransfected in HepG2 cells were examined. As shown in Fig. 3A, as increase the SXR cDNA amount from 25ng to 125ng, the luciferase activity of *phCYP3A4-Luc* was increased and the maximal stimulation was observed when the same amount (100ng) each pSAP-SXR cDNA and *phCYP3A4-Luc* were transfected. Rifampicin treatment was unable to stimulate further the luciferase activity that was increased by pSAP-SXR transfection (Fig. 3A). Newly synthesized HDAC inhibitor, 4-dimethylamino-*N*-[4-(2-hydroxycarbamoylvinyl)benzyl]benzamide (IN2001) was examined whether HDAC inhibitor affect the *CYP3A4* proximal promoter activity when pSAP-SXR and *phCYP3A4-Luc* were cotransfected. As shown in Fig. 3B, 0.5 μM IN2001 significantly increased *CYP3A4* proximal promoter activity over untreated control cells and 1 uM rifampicin concomitant treatment with IN2001 increased further *CYP3A4* proximal promoter activity that was stimulated by IN2001. This data suggested that HDAC inhibitor, IN2001 seemed to facilitate the *CYP3A4* proximal promoter to be activated by chemicals.

HDAC inhibitors increase *CYP3A4* proximal promoter activity

It is known that the degree of acetylation on histones in the promoter region of genes might control the transcriptional activity of promoters and has been reported that histone acetylation affected the induction of the gene expressions of CYPs. To investigate whether a weak response to CYP3A4 inducers in proximal promoter region was related with a chromatin structure, *phCYP3A4-Luc* was cotransfected with pSAP-SXR expression plasmid into HepG2 cells and then treated with 0.05, and 0.5 μM of histone deacetylase inhibitors such as TSA, HC-toxin, IN2001, and IN2002 for 24 h. All of HDAC inhibitors tested increased significantly the luciferase activity driven by *CYP3A4* proximal promoter ($p<0.05$, Fig. 4). 0.05, and 0.5 M TSA increased the promoter activity to 4.9- and 2.3-fold, respectively and it showed a maximum response at 0.05 μM. 0.05, and 0.5 μM HC-toxin increased significantly the luciferase activity to 3.5- and 8-fold, respectively. Also 0.05, and 0.5 M IN2001 increased to 2.5- and 5-fold and IN2002 to 2.8- and 7-fold, respectively. These results indicated that the inhibition of histone deacetylation increased markedly *CYP3A4* proximal promoter activity and thus, a remodeling of chromatin structure might be related with *CYP3A4* stimulation in proximal promoter region.

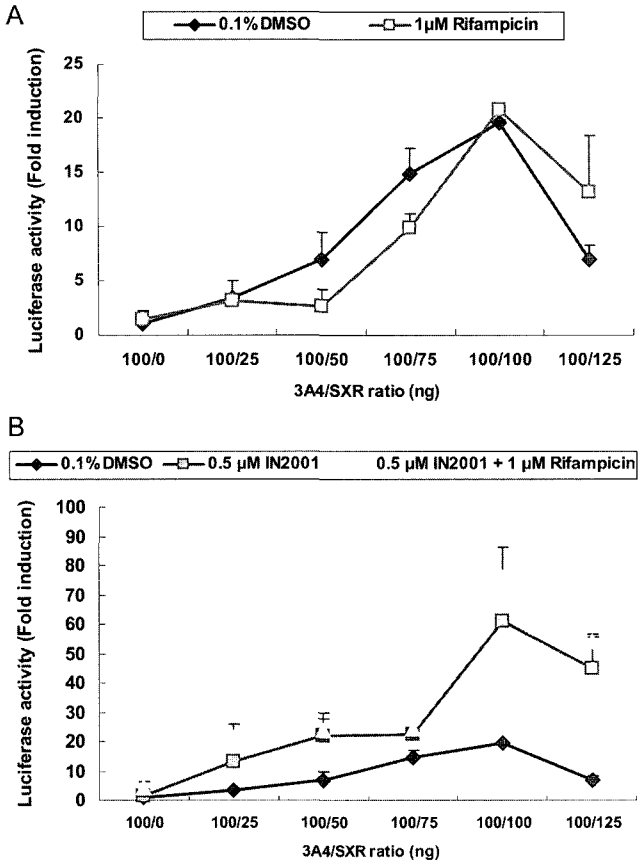

Fig. 3. A. Effects of SXR with CYP3A4 inducer on *CYP3A4* proximal promoter in *phCYP3A4-Luc*/SXR cotransfected HepG2 cells. B. Effects of SXR with HDAC inhibitor and CYP3A4 inducer on *CYP3A4* proximal promoter in *phCYP3A4-Luc*/SXR cotransfected in HepG2 cells. HepG2 cells were transfected by *phCYP3A4-Luc* along with pSAP-SXR. Cells were exposed to chemicals for 24 h. Control cells were treated with 0.1% DMSO. The luciferase activity was measured using luciferin. Data present mean ± S.D. of triplicate transfections from a single representative experiment (Rif : Rifampicin, IN : IN2001). *: significantly different from control at $p<0.05$

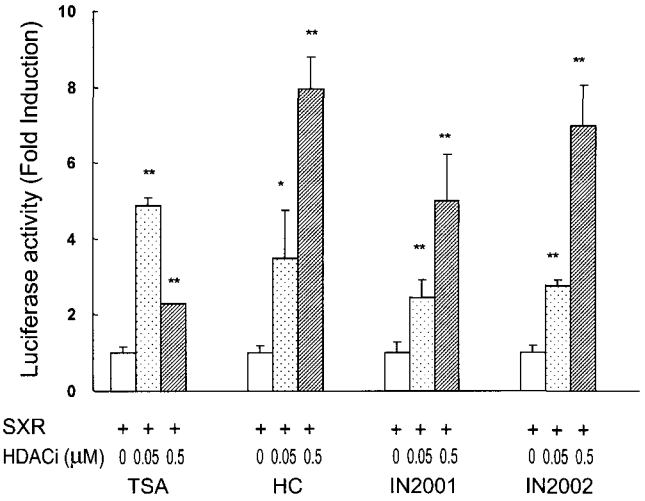

Fig. 4. Effects of HDAC inhibitors on *CYP3A4* proximal promoter in *phCYP3A4-Luc*/SXR cotransfected HepG2 cells. HepG2 cells were transfected by *phCYP3A4-Luc* along with pSAP-SXR. Cells were exposed to various concentrations of HDAC inhibitors such as TSA, HC-toxin (HC), IN2001 and IN2002 for 24 h. Control cells were treated with 0.1% DMSO. The luciferase activity was measured using luciferin. Data present mean ± S.D. of triplicate transfections from a single representative experiment. *: Significantly different from control at $p<0.05$, **: Significantly different from control at $p < 0.01$

TSA effect on *CYP3A4* proximal promoter activity in the absence of SXR

In order to investigate whether TSA affect *CYP3A4* gene expression regulation by CYP3A4 inducers in proximal promoter region, the experiment was performed with HepG2 cells that were transfected with *phCYP3A4-Luc* and were treated with rifampicin, PCN or RU-486 in the presence of 0.05 μM TSA for 24 h. The transfected HepG2 cells were treated with 0.25, 2.5 and 25 μM CYP3A4 inducers and the proximal promoter activity was monitored by measuring the luciferase activity. 0.05 M TSA alone increased the luciferase activity about 5- to 7-fold compared to that of control. When 0.25 μM and 2.5 μM rifampicin were treated with HepG2 cells transfected with *phCYP3A4-Luc*, the luciferase activity was increased 5.1- and 6.7-fold over that of control cells, respectively, while 0.05 M TSA increased 4.7-fold over that of control cells. Concomitant treatment of rifampicin with TSA appeared to increase the luciferase activity more than TSA alone did, although this difference was not statistically significant (Fig. 5). In the case of RU-486, when HepG2 cells transfected with *phCYP3A4-Luc* were treatedRU-486 with 0.25, 2.5 and 25 μM, the activity was increased slightly to 8.9-, 8.9- and 10.8-fold over control cells respectively while 0.05 μM TSA increased 7.5-fold over that of control cells. These results indicated that the CYP3A4 inducers increased further the TSA stimulated proximal promoter activity of *phCYP3A4-Luc* with a dose dependent manner, even although data did not show statistical significance.

TSA effect on *CYP3A4* proximal promoter activity in the presence of SXR

In order to examine the importance of SXR for the CYP3A4 inducing chemicals to be able to regulate the gene expression of *CYP3A4* gene, 0.25 μM to 25 μM CYP3A4 inducers were administered into the HepG2 cells that were transfected with both *phCYP3A4-Luc* and pSAP-SXR in the presence of 0.05 μM TSA for 24 h. 0.05 μM TSA alone increased the luciferase activity about 4- to 6-fold compared to that of control cells. Rifampicin combined with TSA increased the activity significantly in comparison to TSA ($p<0.05$, Fig. 6). When 0.25 and 2.5 μM rifampicin were administered into HepG2 cells that were transfected with both *phCYP3A4-Luc* and pSAP-

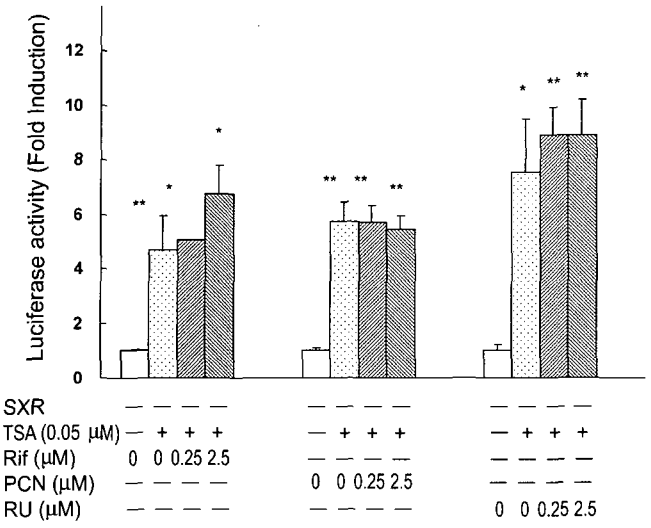

Fig. 5. Effects of TSA on *CYP3A4* regulation by CYP3A4 inducers in *phCYP3A4-Luc* transfected HepG2 cells. HepG2 cells were transfected with *phCYP3A4-Luc*. Cells were exposed to various concentrations of CYP3A4 inducers such as rifampicin (Rif), pregnenolone 16α-carbonitrile (PCN) and RU-486 (RU) in the presence of 0.05 μM TSA for 24 h. Control cells were treated with 0.1% DMSO. The luciferase activity was measured using luciferin. Data present mean ± S.D. of triplicate transfections from a single representative experiment. *: Significantly different from control at $p<0.05$, **: Significantly different from control at $p < 0.01$

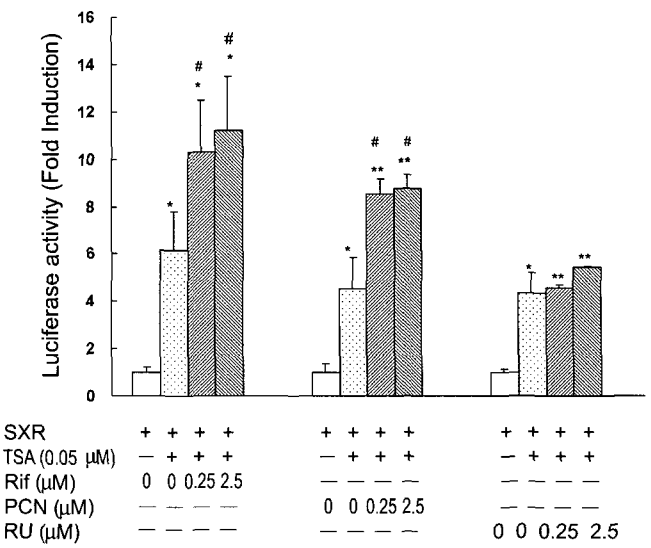

Fig. 6. Effects of TSA on *CYP3A4* regulation by CYP3A4 inducers in *phCYP3A4-Luc* /SXR cotransfected in HepG2 cells. HepG2 cells were transfected by *phCYP3A4-Luc* along with pSAP-SXR. Cells were exposed to various concentrations of CYP3A4 inducers such as rifampicin (Rif), pregnenolone 16α-carbonitrile (PCN) and RU-486 (RU) in the presence of 0.05 μM TSA for 24 h. Control cells were treated with 0.1% DMSO. The luciferase activity was measured using luciferin. Data present mean ± S.D. of triplicate transfections from a single representative experiment. *: Significantly different from control at $p<0.05$, **: Significantly different from control at $p < 0.01$, # : Significantly different from TSA at $p < 0.05$

SXR in the presence of 0.05 μM TSA, the luciferase activity was 10.3- and 11.2-fold increased significantly over the control cells, respectively, with a dose-dependent manner, while 0.05 TSA μM increased 6.2-fold over that of control cells. In the case of PCN, when 0.25, 2.5 and 25 μM PCN were added to the transfected HepG2 cells in the presence of 0.05 μM TSA, the proximal promoter activity was increased significantly to 8.5-, 8.7- and 8.9-fold over the control cells, respectively, with a dose-dependent manner, while 0.05 μM TSA alone treatment increased 4.5-fold over that of control cells. Also, 0.25, 2.5 and 25 μM RU-486 were treated transfected HepG2 cells in the presence of 0.05 μM TSA, the proximal promoter activity was increased significantly to 4.6-, 5.4- and 5.8-fold over the control cells, respectively, with a dose-dependent manner, while 0.05 μM TSA alone treatment increased 4.3-fold over that of control cells. These results demonstrated that the increase of *CYP3A4* proximal promoter activity by CYP3A4 inducers such as PCN, rifampicin, and RU-486 needed both HDAC inhibitor such as TSA and SXR. SXR seems to be important for the dose-dependent response of CYP3A4 inducers to work on *CYP3A4* proximal promoter.

DISCUSSION

Xenobiotic regulation on the *CYP3A4* gene expression has been reported that rifampicin, PCN, and dexamethasone increased the reporter gene activity by *CYP3A4* proximal promoter in HepG2 cells and these increments were enhanced significantly with hGR cotransfection (Ogg *et al.*, 1999). The results of the activity of -1087 to -57 bp of the *CYP3A4* regulatory region showed that 50 μM rifampicin increased the promoter activity to 2.4-fold and 100 μM PCN increased to 1.45-fold (Ogg *et al.*, 1999). Other study demonstrated using the same plasmid as Dr. Ogg did that 50 μM rifampicin increased the luciferase activity to 13-fold and this was enhanced to 26.7-fold by SXR cotransfection (Gibson *et al.*, 2000). These reports are in agreement with our data that SXR enhanced dose-dependent stimulation of *CYP3A4* proximal promoter activity by rifampicin in the presence of HDAC inhibitor, TSA (Fig. 6). In contrast, Goodwin *et al.* reported that 5'-flanking regions of *CYP3A4*, such as -1252 to +53 bp and -362 to +53 bp, were not responded to rifampicin in HepG2 cells and neither they responded to rifampicin plus SXR (Goodwin *et al.*, 1999). Study had showed the difference between hepatic cell line, HepG2 cells and nonhepatic cell lines, such as murine fibroblast, NIH3T3 and simian kidney cells, COS7 in terms of the responsiveness to rifampicin stimulation of *CYP3A4* proximal promoter (-263 to +11 bp) activity in the presence of SXR

(Goodwin *et al.*, 1999; Luo *et al.*, 2002). This suggested that the tissue specific factor(s) might be important for the regulation of *CYP3A4* gene expression by chemical inducers. In the present study, CYP3A4 inducers was unable to activated espression of *phCYP3A4-Luc* containing -863 to +64 bp of the *CYP3A4* proximal promoter without TSA. At this moment, it is not clear whether this nucleotide sequence difference counts for the discrepancy between studies. Importantly, these studies used the potent cytomegalovirus promoter as a minimal promoter, whereas the present study used the native *CYP3A4* proximal promoter. Hashimoto *et al.* demonstrated that CAT activities were scarcely detected, when HepG2 cells were transfected with a few CAT-gene-containing plasmids such as *p2900 CAT* (-2900 to +71 bp), *p1105 CAT* (-1105 to +71 bp) and *p362 CAT* (362 to +71 bp) (Hashimoto *et al.*, 1993). However, their studies with different regions of 5flanking DNA showed that the activity of *p-362 CAT* (-362 to -94 bp) was higher than control vector and the activity of *p1105 CAT* (-1105 to -94 bp) was lower than control vector(Hashimoto *et al.*, 1993). Other study also reported that the activity of luciferase was lower slightly in *p1084 Luc* (-1084 to +53 bp) than control vector when chimeric CYP3A4 luciferase reporters, which contain 5'-flanking regions of *CYP3A4* at the *pGL3-basic* vector, were transfected into HepG2 cells (Goodwin *et al.*, 1999). Thus, an attractive hypothesis related with these results might be that silencer elements might be existed in this proximal region of the *CYP3A4* promoter, possibly -1105 to -362 bp or -57 to +64 bp, and repress a basal transcriptional activity. It was reported that rifampicin treatment neither increased nor decreased the interaction of SXR with SMRT in CV-1 cells (Takeshita *et al.*, 2002). From these results, ligand-dependent interaction of SXR with SMRT in HepG2 cells may require other cell-specific factor or post-transcriptional modification (Takesihita *et al.*, 2002; Synold *et al.*, 2001). It was reported that a corepressor protein, PSF (polypyrimidine tract-binding protein-associated splicing factor), mediated silencing through the recruitment of histone deacetylases (Mathur *et al.*, 2001). Thus the effect of histone deacetylation was examined on transcriptional regulation of the *CYP3A4* gene.

The degree of histone acetylation in the promoter regions of genes appears to control the activity of these promoters by mediating changes in the nucleosomal and chromatin structure of these regions presumably affecting the accessibility of transcription factors to their *cis*-regulatory elements (Wu, 1997; Pazin *et al.*, 1997; Nakajima *et al.*, 2003). However, the mechanism of gene repression or activation is not well understood and might result from either direct or indirect effects of histone acetylation or from the increase in acetylation of proteins other than histones (e.g. transcription factors) (Marks *et al.*, 2003). Gene transcription is repressed by protein deacetylation (Pazin *et al.*, 1997). The present study indicated that the inhibition of histone deacetylation enhanced the *CYP3A4* induction by proximal promoter and affected to a trans-activation of SXR specifically in HepG2 cells. Taking these data into consideration, one could hypothesized that CYP3A4 promoter is repressed by histone deacetylase and TSA inhibits this repression by mediating changes in a chromatin structure and then SXR can interact with *CYP3A4* promoter and eventually mediate stimulations by CYP3A4 inducers (Figs. 3B, 6).

In conclusion, the inhibition of histone deacetylation was required to SXR-mediated stimulation in *CYP3A4* proximal promoter region when rifampicin or PCN were treated in HepG2 cells.

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REFERNECES

Barwick, J. L., Quattrochi, L. C., Mills, A. S., Potenza, C., Tukey, R. H., and Guzelian, P. S., Trans-species gene transfer for analysis of glucocorticoid-inducible transcriptional activation of transiently expressed human *CYP3A4* and rabbit *CYP3A6* in primary cultures of adult rat and rabbit Hepa-Itocytes. *Mol. Pharmacol.*, 50, 10-16 (1996).

Drocourt, L., Ourlin, J. C., Pascussi, J. M., Maurel, P., and Vilarem, M. J., Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human Hepa-Itocytes. *J. Biol. Chem.*, 277, 25125-25132 (2002).

Gibson, G. G., Regulation of the *CYP3A4* gene by hydrocortisone and xenobiotics: Role of the glucocorticoid and pregnane X receptors. *Drug Metab. Dispos.*, 28, 493-496 (2000).

Goodwin, B., Hodgson, E., and Liddle, C., The orphan human pregnane X receptor mediates the transcriptional activation of *CYP3A4* by rifampicin through a distal enhancer module. *Mol. Pharmacol.*, 56, 1329-1339 (1999).

Guengerich, F. P., Cytochrome p-450 3A4: Regulation and role in drug metabolism, *Annu. Rev. Pharmacol. Toxicol.*, 39, 1-17 (1999).

Hashimoto, H., Toide, K., Kitamura, R., Fujita, M., Tagawa, S., Itoh, S., and Kamataki, T., Gene structure of CYP3A4, an adult-specific form of cytochrome P450 in human livers, and its transcriptional control. *Eur. J. Biochem.*, 218, 585-595 (1993).

Itoh, S., Yanagimoto, T., Tagawa, S., Hashimoto, H., Kitamura, R., Nakajima, Y., Okochi, T., Fujimoto, S., Uchino, J., and Kamataki, T., Genomic organization of human fetal specific P-450IIIA7 (cytochrome P-450HFLa)-related gene(s) and

interaction of transcriptional regulatory factor with its DNA element in the 5' flanking region. *Biochim. Biophys. Acta*, 1130, 133-138 (1992).

Ketter, T. A., Flockhart, D. A., Post, R. M., Denicoff, K., Pazzaglia, P. J., Marangell, L. B., George, M. S., and Callahan, A. M., The emerging role of cytochrome P450 3A in psychopharmacology. *J. Clinic. Psychopharmacol.*, 15, 387-398 (1995).

Kocarek, T. A., Schuetz, E. G., Strom, S. C., Fisher, R. A., and Guzelian, P. S., Comparative analysis of cytochrome P4503A induction in primary cultures of rat, rabbit, and human Hepa-Itocytes. *Drug Metab. Dispos.*, 23, 415-421 (1995).

Li, A. P., Kaminski, D. L., and Rasmussen, A., Substrates of human Hepa-Itic cytochrome P450 3A4. *Toxicology*, 104, 1-8 (1995).

Liddle, C., Goodwin, B. J., George, J., Tapner, M., and Farrell, G. C., Separate and interactive regulation of cytochrome P450 3A4 by triiodothyronine, dexamethasone, and growth hormone in cultured Hepa-Itocytes. *J. Clin. Endocrinol. Metab.*, 83, 2411-2416 (1998).

Luo, G., Cunningham, M., Kim S., Burn, T., Lin, J., Sinz, M., Hamilton, G., Rizzo, C., Jolley, S., Gilbert, D., Downey, A., Mudra, D., Graham, R., Carroll, K., Xie, J., Madan, A., Parkinson, A., Christ, D., Selling, B., Lecluyse, E., and Gan, L., CYP3A4 induction by drugs: Correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human Hepa-Itocytes. *Drug Metab. Dispos.*, 30, 795-804 (2002).

Mangelsdorf, D. J., and Evans, R. M., The RXR heterodimers and orphan receptors. *Cell*, 83, 841-850 (1995).

Marks, P. A., Miller, T., and Richon, V. M., Histone deacetylases. *Curr. Opin. Pharmacol.*, 3, 344-351 (2003).

Mathur, M., Tucker P. W., and Samuels, H. H., PSF is a novel corepressor that mediates its effect through Sin3A and the DNA binding domain of nuclear hormone receptors. *Mol. Cell Biol.*, 21, 2298-2311 (2001).

Michalets, E. L., Update: clinically significant cytochrome P-450 drug interactions. *Pharmacotherapy*, 18, 84-112 (1998).

Nakajima, A., Iwanari, M., and Yokoi, T., Effects of histone deacetylation and DNA methylation on the constitutive and TCDD-inducible expressions of the human CYP1 family in MCF-7 and HeLa cells. *Toxicol. Lett.*, 144, 247-256 (2003).

Ogg, M. S., Williams, J. M., Tarbit, M., Goldfarb, P. S., Grays, T. J. B., and Gibson, G. G., A reporter gene assay to assess the molecular mechanisms of xenobiotic-dependent induction of the human *CYP3A4* gene *in vitro*. *Xenobiotica*, 29, 269-279 (1999).

Pascussi, J. M., Gerbal-Chaloin, S., Drocourt, L., Maurel, P., and Vilarem, M. J., The expression of CYP2B6, CYP2C9 and CYP3A4 genes, a tangle of networks of nuclear and steroid receptors. *Biochim. Biophysic. Act.*, 1619, 243-253 (2003).

Pazin, M. J., and Kadonaga, J. T., Whats up and down with histone deacetylation and transcription? *Cell*, 89, 325-328 (1997).

Schuetz, E. G., Schuetz, J. D., Strom, S. C., Thompson, M. T., Fisher, R. A., Molowa, D. T., Li, D., and Guzelian, P. S., Regulation of human liver cytochromes P-450 in family 3A in primary and continuous culture of human Hepatocytes. *Hepatology*, 18, 1254-1262 (1993).

Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P., and Negishi, M., The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J. Biol. Chem.*, 274, 6043-6046 (1999).

Synold, T. W., Dussault, I., and Forman, B. M., The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat. Med.*, 7, 584-590 (2001).

Takeshita, A., Taguchi, M., Koibuchi, N., and Ozawa, Y., Putative role of the orphan nuclear receptor SXR in the mechanism of *CYP3A4* inhibition by Xenobiotics. *J. Biol. Chem.*, 277, 32453-32458 (2002).

Thummel, K. E., Brimer, C., Yasuda, K., Thottassery, J., Senn, T., Lin, Y., Ishizuka, H., Kharasch, E., Schuetz, J., and Schuetz, E., Transcriptional control of intestinal cytochrome P-4503A by 1alpha,25-dihydroxy vitamin D3. *Mol. Pharmacol.*, 60, 1399-1406 (2001).

Wu, C., Chromatin remodeling and the control of gene expression. *J. Biol. Chem.*, 272, 28171-28174 (1997).